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Original Research

Pharmacogenetics of drug metabolizing enzyme: thiopurine methyl transferase phenotypes and multidrug resistance 1 gene polymorphism in inflammatory bowel disease

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Abstract: Inflammatory bowel disease(IBD) is progressing rapidly in developing countries such as Iran. This research is intended to compile the frequency distribution of the drug metabolizing enzyme, thiopurine methyl transferase(TPMT) and the drug transporter, Multi drug resistance(MDR1) which are involved in metabolism of many therapeutics such as thiopurines in inflammatory bowel disease(IBD). Ethnicity is an important variable influencing drug response. The aims of this research were to investigate the association of TPMT phenotypes with MDR1 genotypes. TPMT activity was measured by using a non-extraction HPLC method and genotype for the C3435T polymorphism of MDR1 gene was determined in 215 unrelated IBD patients including of 85 males and 130 females and 212 unrelated healthy individuals consisted of 96 males and 116 females as control group by PCR-RFLP in Iran's western population. TPMT phenotypes demonstrated no frequency for deficient, 2.2% for low and 97.8% for normal activity that is different with results of other studies. Interestingly there were a significant negative correlation between TPMT activities as calculated based on nmol/grHb/h and positive correlation calculated in mU/L with Hb levels in IBD patients and control subjects. Dominant and codominant MDR1 C3435T gene polymorphism increased the risk of IBD by 1.45 and 1.46 times, respectively. IBD patients with MDR1 mutant genotypes C3435T, had lower TPMT activites and Hb concentrations. Using of mU/L is more appropriate than nmol6MTG/grHb/h for expressing TPMT activity. TPMT frequency of deficient and low activity in western Iran is low. The carriers of mutant C3435T MDR1 are not good TPMT methylators.

Key words: Genotype, HPLC, IBD, MDR1, Phenotype, TPMT.

Introduction

Inflammatory bowel disease (IBD) is the term used for a group of diseases, which are chronic and recurrent condition triggered by genetic, environmental, and immunologic factors, increasing almost every-where in the world. The disease was almost non-existent four decades ago in the east, including the middle-east, while nowadays it is seen more and more (1). Ulcerative colitis (UC) and Crohn's disease (CD) (collectively termed inflammatory bowel disease (IBD) are complex disorders reflected by wide variation in clinical practice (2). These diseases, however, were low or non-existent in Iran 50 years ago (3, 4) but are now increasingly growing (5, 6). The main initial goal of treatment in IBD is to induce and maintain remission of disease, and eventually, to achieve mucosal healing and a reduction in the need for hospital admission and surgery. (7, 8). Nowadays, the purines analogues are the most frequently used immunosuppressive drugs in IBD and have proven to be effective in active disease to induce remission, also for the maintenance of clinical remission of both CD and UC. Therefore, thiopurine agents are considered the mainstay of IBD medical therapy, especially in steroid dependent and steroid-refractory patients (9, 10). Thiopurine methyltransferase (TPMT) (EC 2.1.1.67) is a cytosolic enzyme present in most cells of the body. It catalyses the S-methylation of aromatic and heterocyclic sulphydryl compounds such as 6-mercaptopurine(6-MP) and its prodrug Azathiopurine(AZA) (11). This enzyme exhibits variation of its activity and can be measure by commercial laboratories. Approximately 0.3% of the general population has low to absent enzyme activity, 11% has intermediate levels, and 89% has normal to high levels of activity (12). Although thiopurine drugs are widely used, gastrointestinal intolerance, pancreatitis, hypersensitivity and myelosuppression observe in up to 30% of patients. These adverse drug responses (ADR) often result in the withdrawal of treatment (12-14). In some instances the unexpected reaction to therapy can be fatal, for example in cases of severe myelosuppression (15). Although the field of pharmacogenetics dates back to the 1950s, when it was first proposed that inherited traits may account for individual variability in drug response (16) but pharmacogenetics in IBD seems currently to be of immediate scientific as well as of capital clinical impor-

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tance in view of thiopurine therapies. Because it is the linking of differences in gene expression (genotype) to drug response (phenotype) and advances in this field are helping to better predict drug efficacy and toxicity (17, 18).

P-glycoprotein (P-gp), an ATP-binding cassette (ABC) transporter (19) was initially detected as a protein responsible for multidrug resistance in cancer cells. P-gp physiologically is expressed in different human tissues, and probably has several roles. The highest levels of P-gp are found in epithelial cells of the kidney, liver, pancreas, and colon, in cells forming the blood/brain barrier and in lymphocytes. P-gp plays important role in the secretion of toxic compounds (20) in apoptosis (21) and in the immune response (22) The mouse knockout (Mdr1a/) model suggests a role of P-gp in IBD (23). Experiments carried out on mice with the inactive equivalent of the human ABCB1/MDR1 gene (ATP-binding cassette subfamily B member 1; multidrug resistance 1), which encodes multidrug resistance protein (P-glycoprotein), have been shown to develop spontaneous colitis (24, 25). Decreased expression of P-gp is identified in IBD patients as compared to controls (26). Recently, potential functional naturally occurring single-nucleotide polymorphisms (SNPs) within the MDR1 gene are identified in healthy individuals associated with altered duodenal P-gp expression and drug uptake. The extended pharmacogenomics, functional, and complex disease association studies, focused mainly on common synonymous C3435T polymorphism in exon 26, initially associated with lower P-gp functional expression and higher plasma levels of digoxin after oral admission (27). More recently, MDR1 gene expression is significantly reduced in the colonic tissue of UC patients and the C3435T polymorphism was associated with UC (28), but this association was not confirmed in other studies (29). Although population differences and selection of control group for disease association study could play a role in this discrepancy, it is possible that results would be more consistent if an association study was performed with functional SNP(s).

The aim of the present study was to determine the frequency of TPMT activity in the prediction of adverse events, during thiopurine therapy in IBD patients and compare with control group, also evaluate the correlation of these findings with C3435T MDR1 mutation between mentioned groups in western population of Iran.

Materials and Methods

Study Groups

This is a case-control study with Convenience sampling and definite time period. This research was pre-approved by the ethical committee of Kermanshah University of Medical Sciences. Ten ml peripheral blood in ethylene diamine tetra acetic acid (EDTA) (0.5 mM) was obtained from 215 unrelated Iranian IBD patients and 212 unrelated healthy individuals as control group in Mahdieh clinic of Kermanshah Medical University in the period between Februarys to June 2015. Personal and family history was unremarkable. The procedures of the study were approved by the Helsinki research ethics committee of the Iranian authority ministry of health according to the World Medical Association Declaration of Helsinki (30), and a written consent form was obtained from each patient. One of the limitations of this study was the lack of lifestyle and medications information of the participants.

MDR1 genotyping

Genomic DNA was extracted from 5 mL peripheral blood using phenol chloroform extraction method. Primer design and restriction enzyme analysis was performed according to previous studies with minor modifications (31).

The following primers were used for MDR1 point mutation C3435T: forward 5'-TGC TGG TCC TGA AGT TGA TCT GTG AAC-3' and reverse 5'-ACA TTA GGC AGT GAC TCG ATG AAG GCA-3'. PCR reaction consisted of DNA denaturation at 94˚C for 2 min, followed by 35 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, with a final extension at 72˚C for 5 min. After amplification, the products were digested with the restriction enzyme MboI which Wild-type CC genotype showed three fragments (172, 60 and 16 bp); homozygous mutant TT genotype showed two fragments (232 and 16 bp) and heterozygous CT genotype showed four fragments (232, 172, 60 and 16 bp) "Figure 1".

Sample collection, storage, hemolysate preparation and enzyme assay

The substrate S-adenosyl-L-methionine (SAM, A.7007) and 2-amino-6-methyl mercaptopurine (6-MTG, A.9546) and 6-thioguanine (6-TG A. 4882) were obtained from Sigma-Aldrich. A stock standard (50,000 ng/ml) solution of 6-MTG was made by dissolving 5 mg of 6-MTG in 2 ml of 0.1 M NaOH and diluted with distillated water to 100 ml in a volumetric flask. Then we made working standards of 250, 125, 62.5, 31.25 ng/ml with serial dilution.

The incubation mixture comprised final concentrations of 600 µmol/L 6-TG and 80 µmol/L SAM. This was made up by dissolving 6-TG (14 mg) in 4 ml of

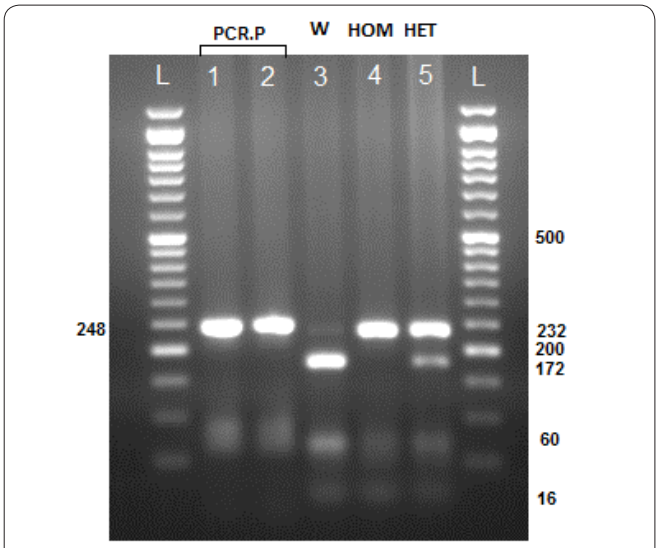

Figure 1. Electrophoresis patterns for MDR1 C3435T alleles analyzed by PCR-RFLP. L, 50 bp DNA ladder; PCR.p, PCR product; W, homozygous wild-type (CC); HOM, homozygous mutant (TT); HET, heterozygous mutant (CT). The expected PCR fragments were detected using 2.5% agarose gel electrophoresis.

0.1 M NaOH. SAM (4.2 mg) was dissolved in 50 ml potassium phosphate buffer (0.1 mol/l. pH 7.4). The two substrates were mixed together and made up to a final volume of 100 ml with 0.1 mol/l potassium phosphate buffer (pH 7.4). The substrate mixture (0.5 ml) was then pipetted into incubation tubes, which were capped and stored at -70 ◦C for up to 1 month prior to use.

Apparatus and chromatographic conditions

The chromatographic apparatus consisted of an Agilent technologies model 1200 series quaternary pump (Germany). The stationary phase was a MZ analytical 5µ reverse-phase column (C 18, 150 X 4.6 mm Germany) at ambient temperature protected with a 4mm x 3mm security guard column (Phenomenex, UK). The mobile phase (flow-rate, 2ml/min) was an isocratic solution consisted of water and methanol (80:20 v/v) and 100mM of triethylamine (TEA) which final pH was adjusted to 6.2 with concentrated ortho phosphoric acid. The fluorescence detector was set at excitation 315 nm and emission 390 nm.

Incubation tubes were 2 ml screw top plastic vials. Reaction tubes were incubated in a Memmert (Germany) water bath and finally in a dry plate incubator Kiagen (Iran). The haemoglobin content of whole blood was determined by Zist Shimy (Iran) hemoglobin kit.

Sample collection, storage and hemolysate preparation

Blood samples were collected into EDTA tubes and can be stored at 4◦C for a maximum of 2 days at 4◦C prior to analysis. The whole blood samples were mixed well by inversion and two 200 µl aliquots were accurately pipetted into test tubes and capped. The tubes were frozen for 15 minutes at -80◦C to disrupt the cells without the need of lysate solution. The cells were shock thawed by the addition of 600 µl of whole blood suspension buffer (0.1 M KH2PO4 titrated with 0.1 M K2HPO4 (Sigma-Aldrich) to pH 7.4), then vortex mixed.

Incubation and HPLC analysis

The stored incubation tubes cotaining the buffer and substrate mixture were pre-warmed in the water bath at 37◦C for 5 min and 200µl of lysate added to tubes at 15-second intervals. Tubes were incubated for exactly 1 h, then the reaction was stopped by transferring tubes to the dry plate incubator for 10 min at 90◦C. Samples were cooled in cold water and centrifuged at 1400 g for 5 min. The supernatants were transferred to another tubes and 20 µl injected to the loop.

Standardisation and blank

A standard curve was shown to be linear over the range 0 - 2.76 nmol 6-MTG with the equation of the curve being y=0.0369x + 0.0717, (R=1). A standard blank was prepared by adding 200 µl potassium phosphate buffer (0.1 mol/l. pH 7.4) to incubate. A sample blank was prepared by adding 200 µl of lysate and then stopping the reaction at time zero. The 6-MTG component elutes from the column as a sharp narrow peak that is completely isolated from the rest of the components. The retention time of 6-MTG (5.75 min) on the column is specific to the compound and the peak height is exactly proportional to its concentration. The chromatogram of the 6-MTG standard, reaction blank, low, normal and high TPMT activities are presented in "Figure 2".

Ethical Considerations

The human subject study protocol was approved by the Ethics Committee of the Kermanshah University of Medical Sciences (KUMS), Iran and was in accordance with the principles of the Declaration of Helsinki II and all subjects provided written informed consent.

Statistical analysis

The allelic frequencies were calculated by the gene counting method. The $\chi 2$ test was used to verify the agreement of the observed genotype frequencies with those expected according to the Hardy–Weinberg equilibrium. The MDR1 genotypes frequency in IBD patients were compared to controls using the $\chi 2$ test. Data were analyzed first for normality of distribution by using the Kolmogorov–Smirnov test. Results were expressed as mean±SD for normally distributed data, median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data. Comparison of groups was carried out with Student's

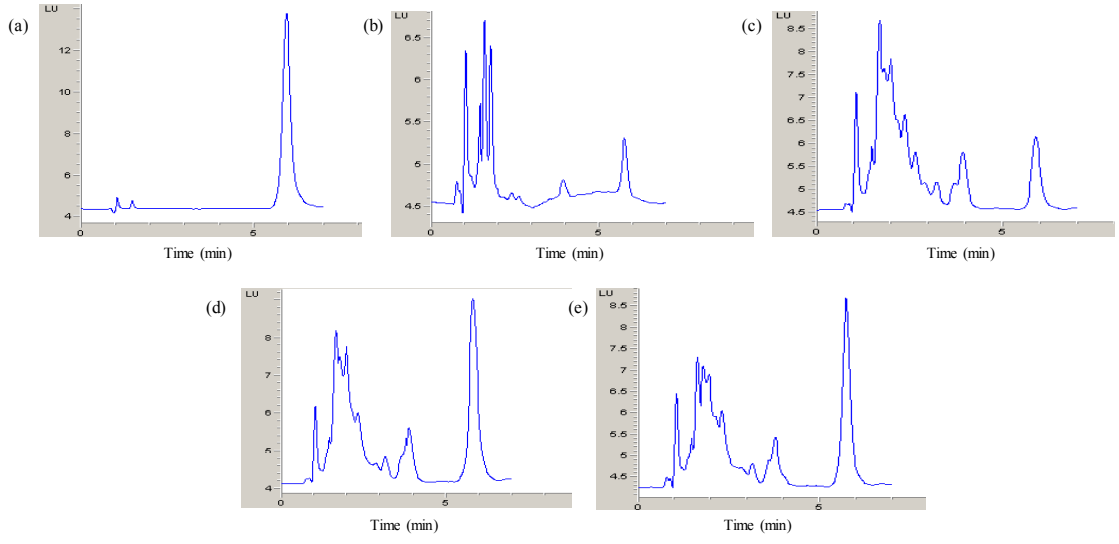

Figure 2. High performance liquid chromatography of 6-Methyl thioguanine. (a) Standard (250ng/mL or 1.37nmoL/mL or 23 mU/L), (b) Reaction blank, (c) Low activity patient (44 mU/L), (d) Normal activity individual (133 mU/L), (e) High activity individual (156 mU/L).

Table1. The demographic characteristics and distribution of the thiopurine methyltransferase (TPMT) activities and other risk factors in inflammatory bowel disease (IBD) patients and control group in a population from Kermanshah province.

	IBD patient (n=215)	Control subjects (n=212)	p values
Age (years)	35.9 ±13.2	34 ±14.2	0.58
Sex (M/F)	86/129	96/116	0.27
TPMT activity (nmol6MTG/grHb/h)	*46.5(40.9 -53.8)	45(41.2 -50.6)	0.026
TPMT activity (mU/L)	108.9±20.1	111.7±19.8	0.15
Hb (g/dL)	13.8±1.85	14.6±1.73	<0.001
BMI (Kg/m^2)	24±3.97	24.1±4.51	0.87

6MTG =6methylthioguanine

*Median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data.

t test, Mann–Whitney U test and one-way ANOVA as appropriate. The correlation values of TPMT activities in mU/L and in nmol/grHb/h, Hb levels, BMI and age of IBD and control groups were calculated using Pearson correlation.

Comparison of TPMT activities in mU/L and nmol6MTG/grHb/h and Hb concentration between TPMT wild and mutant genotypes in total study samples were calculated using two-tailed Student's t test. Statistical significance was assumed at p<0.05. The SPSS statistical software (SPSS for Windows 16; SPSS Inc, Chicago, IL, USA) was used for the statistical analysis.

Results

The clinical, laboratory and demographic characteristics of participants are summarized in Table 1.

There was no significant difference between the mean of TPMT activity in mU/L, age, BMI, Hb and sex of the two groups. However, when TPMT was calculated in nmol6MTG /grHb/h, the concentration of TPMT in IBD patients was false-fully significantly higher than control group (46.5(40.9 -53.8) vs 45(41.2 -50.6), p=0.026, respectively).

Correlations between TPMT activities in mU/L and in nmol6MTG/grHb/h with Hb concentration, BMI and age in IBD patients and control group are shown in Table 2.

There was a significant negative correlation between TPMT activities as calculated based on nmol 6MTG/grHb/h and the Hb levels in IBD and control groups (r = - 0.54, p<0.001 and r = - 0.27, p<0.001), respectively. Interestingly a significant positive correlation between Hb levels and TPMT activities were seen when the activity calculated in mU/L in IBD patients and control subjects (r = 0.14, p=0.05 and r = 0.43, p<0.001), respectively.

Comparison of clinical, laboratory features and risk factors in subgroups of IBD patients and control groups are demonstrated in Table 3.

IBD patients and control group were divided into two subgroups based on Hb concentrations less than 12 and ≥12 gr/dL. As expected, in both groups of IBD patients and control with Hb levels <12 gr/dL had false-fully significantly higher TPMT activities in nmol6MTG/grHb/h compared with both groups of IBD patients and control with Hb levels≥12 gr/dL [57.2(54.7-68) vs 45.4(40.5 – 50.1). p<0.001, and 55.6(48.8-60) vs 44.6 (40-49.3); p<0.001] respectively. So It can be predicted, in both groups of IBD patients and control with Hb levels less than 12 gr/dL compared with both groups with Hb concentrations ≥12 gr/dL would have lower TPMT activity in mU/L (101.9 vs 110.; p=0.041, and 101 vs 112.3.; p=0.036), respectively.

The frequency of multidrug resistant MDR1 C3435T genotypes and alleles in IBD patients and control group are shown in Table 4. As shown in this table, the overall distribution of the MDR1 C3435T genotype in IBD patients had a significantly trend to different from that of the control group (χ2=5.03, df=2, p=0.081). However,

Table 2. Correlation of TPMT activities in mU/L and nmol6MTG/grHb/h with Hb concentration, BMI and age in IBD patients and control group.

	IBD patients (n=215)					Control group (n=212)				
	TPMT activity (mU/L)	TPMT activity (nmol6MTG/ grHb/h)	gr/Hb	BMI	Age	TPMT activity (mU/L)	TPMT activity (nmol6MTG/ grHb/h)	gr/Hb	BMI	Age
TPMT activity (mU/L)	r=1	r=0.72, p<0.001	r=0.14 P=0.05	r=0.03 P=0.7	r=0.05 P=0.5	r=1	r=0.73, p<0.001	r=0.43 p<0.001	r= -0.07 p=0.9	r= -0.15 p=0.029
TPMT activity (nmol6MTG/ grHb/h)	r=0.72, p<0.001	r=1	r= -0.54 P<0.001	r=-0.08 P=0.3	r= -0.07 P=0.34	r=0.72, p<0.001	r=1	r= -0.27 p<0.001	r= -0.02 p=0.77	r= -0.08 p=0.26
Hb gr/dL	r=0.14 P=0.05	r= -0.54, P<0.001	r=1	r= 0.12 P=0.1	r= -0.04 P=0.54	r= 0.43 p<0.001	r= -0.27, p<0.001	r=1	r= 0.03 p=0.7	r= -0.07 p=0. 3
BMI (Kg/m^2)	r=0.03 P=0.68	r=-0.08 P=0.3	r= 0.12 P=0.1	r=1	r= 0. 3 P<0.001	r= -0.01 p=0.9	r= -0.02, p=0.77	r= 0.03 p=0. 7	r=1	r= 0.4 p<001
Age (year)	r=0.05 P=0.5	r=0.07 P=0.34	r= -0.04 p=0.6	r= 0. 3 P<0.001	r=1	r= -0.15 p=0.029	r= -0.08 p=0.26	r= -0.07 p=0. 3	r= 0.4 p<001	r=1

Table 3. Comparison of TPMT activities in mU/L and nmol6MTG/grHb/h, Hb concentration and BMI between subjects with Hb concentration less than and more than 12 gr/dl in IBD patients and control group separately.

	Control group (n=212)		IBD patients (n=215)	
	Hb < 12 gr/dl N=13	Hb ≥ 12 gr/dl N=199	Hb < 12 gr/dl N=30	Hb ≥ 12 gr/dl N=185
TPMT activity (mU/L)	101 ±12.4	112.3±19.9 P=0.036	101.9 ±20.2	110±19.6 p=0.041
TPMT activity (nmol6MTG/grHb/h)	*55.6 (48.8-60)	*44.6 (40-49.3) p<0.001	*57.2(54.7-68)	*45.4(40.5 – 50.1) P<0.001
Hb gr/dl	*12.4 (11.6-12.75)	* 15 (14.2-16) p<0.001	*12.1(11.2-12.5)	* 14.4(13.7-15.6) P<0.001
BMI (Kg/m^2)	23.7±3.4	24.1±4.6 p=0.78	23.1±3.8	24.3±4 p=0.15

*Median and interquartile range (IQR) for non-normally distributed data, and percentages for categorical data.

Table 4. Odd ratio and distribution of multidrug resistant MDR1 C3435T genotypes and alleles in patients with IBD and control subjects.

	IBD patients (n=210)	Control subjects (n=212)
MDR1 C3435T genotypes		
Wild	40 (19%)	54 (25.5%)
Hetero	113 (53.8%) (χ2=4.6, df=2, p=0.033)	105(49.5%)
homo	57 (27.1%) (χ2=3.5, df=2, p=0.062)	53 (25%)
	(χ2=5.03, df=2, p=0.081)	
	IBD patients reference group OR (95%confidential interval)	Control reference group
Codominant		
Homo vs. wild	1.45 (0.98–2.15, p=0.062) (n=57 vs. n=40)	(n=53 vs. n=54)
Hetero vs. wild	1.46 (1.03–2.05, p=0.034) (n=113 vs. n=40)	(n=105 vs. n=54)
Dominant		
Homo + Hetero vs. wild	1.45 (1.05–2.02, p=0.025) (n=170 vs. n=40)	(n=158 vs. n=54)
MDR1 alleles		
Wild	n=193(46%)	n=213(50.2%)
Mutant	n=227(54%)	n=211(49.8%)
	1.1 (0.99-1.2, p=0.078) (χ2=3.1, df=1, p=0.07)	

hetero genotype of MDR1 C3435T had significantly higher frequency in IBD patients compared with control group. The age and sex adjusted OR indicated that both dominant (TT + TC vs. CC), and co dominant (TC vs. CC) MDR1 C3435T gene polymorphism significantly increased the risk of IBD by 1.45 and 1.46 times, respectively.

We analyzed the relation of MDR1 C3435T genotypes (TT + TC vs. CC) with TPMT activities in mU/L and in nmol6MTG/grHb/h, Hb concentration and BMI. As shown in Table 5, IBD patients with one or two copies of mutant genotypes (TT + TC vs. CC) of MDR1 C3435T, had significantly lower TPMT activity in mU/L (p=0.015) and Hb gr/dL (p<0.001) concentration and higher TPMT activity in nmol6MTG/grHb/h (p=0.042) compared with control subjects with the same genotypes of MDR1 C3435T.

In addition, we investigated correlation of TPMT activities in mU/L, nmol6MTG/grHb/h with Hb concentrations, in dominant model of MDR1 C3435T genotypes in IBD patients and control group, separately in Table 6. In both groups of IBD patients and control in dominant model of MDR1 C3435T genotypes there is a significant positive correlation between Hb levels with TPMT activity in mU/L (r=0.16, p=0.043 and r=0.48, p<0.001, respectively), however there is a significant negative correlation between Hb levels with TPMT activities in nmol6MTG/grHb/h (r= -0.52, p<0.001 and r= -0.24, p=0.003, respectively).

Discussion

This study was designed: (i) to evaluate the frequency distribution of TPMT phenotypes and (ii) to identify the possible effects of genetic variants of MDR1 on TPMT activity and pathogenicity of IBD cases in wes-

Table 5. Comparison of TPMT activities in mU/L and nmol6MTG/grHb/h, Hb concentrations and BMI between dominant models of MDR1 C3435T in IBD patients with control subjects.

	IBD patients (n=57)	Control subjects (n=53)	p values
MDR1 C3435T genotypes	TT	TT	
TPMT activity (mU/L)	111±20	110±19.4	0.78
TPMT activity (nmol6MTG/grHb/h)	48.8±10.6	44.3±8.4	0.024
Hb gr/dl	13.7±1.9	14.8±1.8	0.007
BMI (Kg/m^2)	25±3.5	24.1±72	0.7
	CC +TC	CC +TC	
TPMT activity (mU/L)	108.5±20.2	112.6±19.6	0.015
TPMT activity (nmol6MTG/grHb/h)	47.2±10.6	45.8±7.2	0.042
Hb gr/dl	13.8±1.8	14.6±1.7	<0.001
BMI (Kg/m^2)	23.9±4	24±4.2	0.78

Table 6. Correlation of TPMT activities in mU/L, nmol6MTG/grHb/h with Hb concentrations, in dominant model of MDR1 genotypes in IBD patients and control group, separately.

	MDR1 TT genotype IBD patients (n=57)		MDR1 TT genotype Control group (n=53)	
	TPMT activity (mU/L)	TPMT activity (nmol6MTG/grHb/h)	TPMT activity (mU/L)	TPMT activity (nmol6MTG/grHb/h)
Hb gr/dl	r=0.06, p=0.6	r= -0.62, p<0.001	r=0.32, p=0.027	r= -0.373, p=0.006
	CC + TC genotypes of MDR1 IBD patients (n=170)		CC + TC genotypes of MDR1 Control group (n=158)	
	TPMT activity (mU/L)	TPMT activity (nmol6MTG/grHb/h)	TPMT activity (mU/L)	TPMT activity (nmol6MTG/grHb/h)
Hb gr/dl	r=0.16, p=0.043	r= -0.52, p<0.001	r=0.48, p<0.001	r= -0.24, p=0.003

tern population of Iran.

To the best of our knowledge, this is the first analysis of phenotype correlation of the TPMT enzyme with MDR1 C3435T polymorphism performed in IBD patients and healthy individuals with the same ethnic origin of western Iran. The commonly accepted frequency distribution of TPMT deficiency is 1 in 300 (0.3%) individuals, at low activity in another 11% of the population and is normal in approximately 89% individuals. In our study, this clearly defined trimodal frequency distribution of TPMT was not demonstrated, which is not in line with the first description of TPMT polymorphism in 298 White Americans by Weinshilboum and Sladek (12). Surprisingly we found a bimodal distribution with the frequency of intermediate (2.2%) and normal metabolizers (97.8%) which was different to previous reports. Therefore, the risk of toxicity is much lower after utilizing thiopurine drugs for IBD patients in this region.

We have used a non-extraction isocratic HPLC method to determine the concentration of 6-MTG produced to assess TPMT enzyme activity in erythrocytes. This modified robust assay is suitable for use in the routine clinical laboratory to predict the risk of thiopurine toxicity in (IBD) patients prior to receiving thiopurine drugs.

Although majority of the centers around the world, are using the TPMT activity in relation to hemoglobin or RBC content of the blood cell lysate to determine the toxicity of thiopurine drugs, we found a significant negative correlation between TPMT activity in nmol6MTG/grHb/h with the Hb levels in both study groups. Graham have shown that the expressing patient whole blood activity as mU/L will give a result which classifies patients equally well against their underlying genotype (32).

In addition, we found that correlation of TPMT activity with toxicity of thiopurine drugs in IBD patients is significantly affected by hemoglobin level. TPMT activity is significantly greater in IBD than control group when it is calculated in nmol6MTG/grHb/h, whereas, when TPMT activity is calculated in mU/L no statistically significant differences were seen between the two study groups. The observed difference is due to a decrease in hemoglobin level in IBD patients compared to control group. In support of this theory we divided IBD patients and control group into two subgroups based on Hb concentrations less than 12 and ≥12 gr/dL. Also we noticed that in some patients with low hemoglobin level, misleading high results can be obtained and this was not seen when results are expressed as mU/L TPMT. For encouraging other providers to move to mU/L reporting format and expression into routine use we need further presentations.

MDR1 gene encodes P-glycoprotein, a transmembrane efflux pump transferring both exogenous and endogenous substrate from the cells which the single nucleotide polymorphism at position 3435 is considered to be one of the most significant ABCB1/MDR1 gene mutations. Since P-glycoprotein is regarded as a protective barrier, altered P-gp expression and function in the gastrointestinal tract due to this mutation could affect the uptake of xenobiotics (27). There is also a hypothe-

sis that the 3435T variant is able to alter the MDR1 expression by impact on mRNA splicing, its stability or translation efficiency (33, 34). Nevertheless, the mechanism of possible functional effect of the C3435T polymorphism remains not known. Thus, analyzing the C3435T polymorphism may influence encoding P-gp and appears to be of great prognostic importance while evaluating individual susceptibility to IBD.

Two separate studies which have performed in Iranian population demonstrated controversial results of the C3435T genotype in association with the risk of IBD development.

Farnood et al. observed in Tehran population a higher risk of IBD development for the 3435T allele carriers ($p < 0.001$, OR = 1.52) and the 3435TT homozygote's ($p = 0.044$, OR = 1.62) (35).

In another investigation in Iranian Azeri Turks population Bonyadi et al. showed that patient group had a higher frequency of CC Genotype compared to control group (22.4% vs. 19.5%) (36). Although their results were in accordance with the study of this polymorphism in German population, which indicated a higher frequency of the CC genotype in patients with IBD than in healthy individuals (29.2% vs. 22.2%) (37) but were In contrast with the previous Farnood´s study that indicated higher frequency of TT Genotype in IBD patients compared to controls ($P = 0.044$, OR = 1.62) (35).

In present study we found that the overall distribution of the MDR1 C3435T genotype in IBD patients had a trend to different from that of the control group ($\chi2=5.03$, df=2, $p=0.081$). However, hetero genotype (CT) of MDR1 had significantly higher frequency in IBD patients compared with control group (53.8% vs 49.5%, $\chi2=4.6$, df=2, $p=0.033$). The age and sex adjusted OR indicated that both dominant (TT + TC vs. CC), and codominant (TC vs. CC) MDR1 C3435T gene polymorphism significantly increased the risk of IBD by 1.45 and 1.46 times, respectively. Our results also support the previously described role of MDR1 C3435T polymorphism and intestinal P-glycoprotein expression in susceptibility to IBD (28, 38). So we can conclude that the 3435T polymorphism of the ABCB1/MDR1 gene may be a risk factor for IBD in the population coming from western Iran.

Interestingly we found that IBD patients with dominant mutant genotypes of MDR1 C3435T, had significantly lower TPMT activity in mU/L ($p=0.015$). Since MDR1 P-glycoprotein is regarded as a protective barrier, altered P-gp expression and function in the gastrointestinal tract due to this mutation could affect the uptake of xenobiotics and this may have an effect on enzyme activity. It seems more researches are necessary for its confirmation.

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